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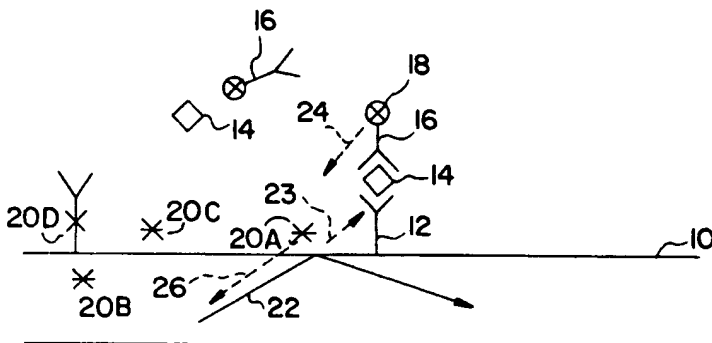
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(54) **Multiple output referencing system for evanescent wave sensor.**

(57) An evanescent wave system and method including an optical sensor for use in assaying a reference material and at least one molecular species or analyte in a test medium or test sample for diagnostic and other applicable purposes. The sensor includes a waveguide for propagating a radiation input along its length. The radiation input causes evanescent electromagnetic waves that are capable of stimulating output emissions that are indicative of a reference material and of one or more molecular species or analytes. By comparing the emission(s) indicative of the reference material to the emission indicative of the presence of the molecular species or analyte, the presence and concentration of the molecule in the sample can be determined. The reference material provides for normalization and/or calibration of the system.



**FIG. I**

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FIELD OF THE INVENTION

This invention relates to an evanescent wave system; including an optical processor, sensor, for use in assays of one or more analytes or molecules in a test medium, and a reference material, and more particularly, to such a sensor which generates two or more output signals from one or more wave propagating surface. The reference material provides for normalization and/or calibration of the system.

TECHNICAL DISCLOSURE

There are a number of optical devices which propagate radiation by "total internal reflection", see Harrick, N.J., Internal Reflection Spectroscopy, Harrick Scientific Corp., Ossining, N.Y. (Third printing 1987), to generate an evanescent wave at the interface of the device and a medium or test sample having a lower index of refraction. The evanescent wave is an electromagnetic waveform which typically extends less than a wavelength into the test medium. However, this penetration is sufficient to permit interaction between the evanescent wave component and an analyte in the medium.

One example of such devices is the use of waveguides in the area of fluorescent immunoassays. Waveguides in the form of fiber optic waveguides are coated with a reactant coating including for example either an antibody or an antigen, which binds the corresponding antigen or antibody, respectively, if present, in a medium. This reactant coating is typically attached to the waveguide prior to the performance of the assay. In a "sandwich" immunoassay, an antibody is coated to the surface of the fiber optic waveguide to form a reactant coating, and the waveguide is contacted with a medium believed to contain the antigen (analyte) to be analyzed, and an added second antibody, previously labelled with a fluorescent molecule. Alternatively, in a "competitive" assay, the fluorescently labelled antigen is first mixed with the medium suspected of containing an antigen and the mixture is brought into contact with the reactant coated waveguide. In either technique, the coated antibody binds with the antigen to form a complex attached to the fiber optic waveguide.

Electromagnetic radiation is introduced into the fiber optic waveguide at one or more predetermined sets of wavelength bands and is propagated along the waveguide by "total internal reflection". The reflection is, of course, not completely total as, for example, a fluorescent molecule absorbs a small amount of evanescent radiation.

The attached fluorescent molecule (referred to as a fluorophore) absorbs energy from the evanescent wave at a first set of wavelengths and fluoresces at a second, longer set of wavelengths. Fluorescence from the excited fluorophore passes into the optical waveguide via a tunneling effect and the portion of the fluorescent radiation which occurs at an angle greater than the critical angle is propagated through the waveguide to emerge as an output signal.

Several improved waveguides are described in U.S. Patent No. 4,880,752; which is incorporated herein by reference. In one construction, the waveguide has an elongated, rod-shaped core having an opening within the core material. A reactant coating is disposed about the opening within the core on a single optical surface.

There is, however, a need for providing an evanescent sensor which can normalize or calibrate for variations in signal input and signal collecting efficiencies as well as defects in the optical surface or geometry. For example, a variation in diameter of a solid rod or of wall thickness of a hollow fiber optic waveguide changes the angle of light input at which light is totally internally reflected. This changes the evanescent wave signal and may cause the radiation to eventually strike at an angle less than the critical angle and therefore escape the waveguide. Alternatively, if the angle becomes increasingly greater than the critical angle, there will be fewer reflections and a lower propagation angle, both of which reduce the amount of evanescent wave electromagnetic field strength which is delivered to a medium.

Another problem concerns irregularities in the wave propagating surface of the sensor, altering the amount of signal output detected.

SUMMARY OF THE INVENTION

In a first embodiment of the invention, analysis of both one or more analytes and a reference material is achieved by attaching a reactant coating and a reference material onto an evanescent sensor, and in a preferred embodiment on the same wave propagating surface from which the reference material and analyte(s) are sensed in the evanescent zone. The reference material functions to normalize and/or calibrate the system. Alternatively, the reference material may be physically independent of the sensor and yet still function to normalize and/or calibrate the system.

In use, the invention allows output signals from both the reference material and the analyte to be detected so as to provide a means for normalizing and calibrating the system for any variations in the sensor and instrument to sensor coupling efficiency which affect the signal input and signal collection. See Harrick, *infra*, and Love, W.F., Bulton, L.J., and Slovacek, R.E., "Optical Characteristics of Fiber Optic Evanescent Wave Sensors" printed in Biosensors With Fiberoptics, D. Wise and L. Wingard, Editors, Humana Press, Clifton, N.J.; both of which are incorporated herein by reference. Since any geometric variations or surface irregularities in the sensor will affect the known output signal from the reference material in the same manner as the signal from the analyte, a ratio of the output of the analyte signal to the output of the reference signal provides a value that will vary only with differences in the analyte and not with differences between sensors. To avoid difficulty in the analysis of the output signals, the reference material must be selected to provide an output signal wavelength that is different and/or readily distinguishable from the signal that is indicative or representative of the analyte. The reference material is used for signal normalization and/or calibration of the system in order to provide accurate and precise test results; and may be measured prior to, during or after the analyte measured signal.

Further advantages and objectives of the reference material include testing the effects of aging on the reactant coating; correlation of substrate and coating variability between sensors in a given manufacturing batch, lot, or assay; and examining variations due to sensor manufacture or handling during coating. The use of the reference material further provides the advantages of detecting defective sensors i.e. a waveguide having a wave propagating surface irregularity, including for example a scratch or chip.

In a second embodiment of the invention, multiple analytes can be sensed or detected using a single sensor. One or more wave propagating surfaces may be utilized for various assay formats. In one example of this embodiment, the test sample to be analyzed will contain one or more antibodies for analyte antigens, in which each one or more of the antibodies are labelled with fluorophores that emit different output wavelengths or signals on exposure to the evanescent wave. If determination of only the relative amount of the individual analyte is desired, this value can be directly determined by comparing the individual output signals. Alternatively, if an absolute analyte value is desired, a reference material, as described above, can in one embodiment of the invention be incorporated onto the same wave propagating surface from which the analyte is sensed. The reference material must be selected to provide an output signal wavelength that is either the same or different than that of the analyte.

In a third embodiment of the invention, an assay can be performed in which the two analyte fluorophores overlap in their wavelength absorption and emission bands; provided that proper fluorescent molecules are selected for the individual analytes.

In a fourth embodiment of the invention the reference material is added to the test medium believed to contain the analyte to be detected, and the evanescent reaction carried out as described above.

In a fifth embodiment of the invention, the reference material is used as a reference material for itself. In an example of this embodiment, a fluorophore reference material is used as a reference for itself. This embodiment provides the advantage of a reduced signal to noise ratio vs. a two fluoromer system.

Each embodiment of the invention is operable in a system including an optical processor.

#### 40 OBJECTS OF THE INVENTION

It is among the objects of the invention to provide an improved evanescent wave system where the system's apparatus includes a sensor, for use in assays of analytes or molecules in a medium or test sample and a reference material, and more particularly, to such a sensor which generates two or more output signals from one or more wave propagating surfaces. The reference material provides for normalization and/or calibration of the system.

A primary object of the invention is to provide an optical processor for a multifrequency evanescent wave system.

Another object of the invention is to provide an evanescent wave sensor having two or more fluorophores.

Yet another object of the invention is to provide an evanescent wave system including a sensor and a reference material, the reference material functioning to normalize and/or calibrate for variations in the sensor and instrument to sensor coupling efficiency which affect the signal input and signal collection.

A still further object of the invention is to provide a sensor that allows at least one analyte and a reference material to be analyzed by a wave propagating surface.

Another object of the invention is to provide an evanescent wave system which can compensate for variations between different sensors.

Yet another object of the invention is to provide an evanescent wave system including a sensor that

provides accurate output signals that are corrected for changes in those things which cause signal variations by the sensor.

Another object of the invention is to provide a sensor incorporating a reference material.

## 5 DESCRIPTION OF THE DRAWINGS

The foregoing and other objects and advantages of the invention will be appreciated more fully from the following further description thereof with reference to the accompanying drawings wherein:

Fig. 1 is a schematic cross-sectional view of a portion of an evanescent wave sensor having a single analyte coated thereon and showing various formats of interactions of the reference material at the evanescent zone.

Fig. 2 is a schematic cross-sectional view of a portion of an evanescent wave sensor having a reference material and two analytes coated thereto.

Fig. 3 is a schematic cross-sectional view of a portion of a cylindrical evanescent wave sensor having a reference material and two analyte binding partners bound to the surface via avidin.

Fig. 4 is a schematic cross-sectional view of a portion of a cylindrical evanescent wave sensor having a reference material encapsulated in a coating on the sensor.

Fig. 5A is a chart of intensity versus wavelength showing overlapping absorption or excitation wavelengths and two different emission wavelengths.

Fig. 5B is a chart showing two fluorophores having different absorption and emission spectra.

Fig. 5C is a chart showing overlapping spectra of the emission wavelength of a first fluorophore and the absorption wavelength of a second fluorophore.

Fig. 6A is a schematic cross-sectional view of an evanescent wave sensor having a diameter that increases in the distal direction.

Fig. 6B is a schematic cross-sectional view of an evanescent wave sensor having a diameter that decreases in the distal direction.

Fig. 7 is a schematic cross-sectional view of an evanescent wave sensor having a surface defect.

Fig. 8 is a schematic representation of a system according to the invention.

Fig. 9 is a schematic representation of another system according to the invention.

Fig. 10 is a schematic representation of another system according to the invention.

Fig. 11 is a schematic representation of another system according to the invention.

## DESCRIPTION OF THE ILLUSTRATIVE EMBODIMENT

An evanescent wave system is described including a sensor, reference material and operation means according to this invention and method for using the system. An optical processor is a component of the system. In one embodiment, the sensor comprises two planar parallel surfaces, whereas in a preferred embodiment, the sensor comprises a waveguide having a wave propagating surface, as in the case of a fiber optic.

The sensor receives a radiation input and emits one or more output signals indicative of the presence of one or more analytes and a reference material in the evanescent wave region (Harrick, *infra* and Love et al *infra*). In the case of a waveguide, the wave propagating surface is capable of receiving a radiation to excite a reference material, the same radiation, if desired, also exciting at least one analyte or a second sequential or simultaneous radiation to excite at least one analyte complex. The sequence of introducing radiation to excite the reference material or analyte may be varied as necessary in the practice of various test methods.

In the case of the sensor having two separate parallel, planar surfaces, the input light is propagated between the two surfaces. A single surface is capable of receiving at least a first radiation input and generating a signal output indicative of the presence of a reference material as well as a radiation output signal indicative of the presence of at least one analyte.

One characteristic of the invention is that the evanescent wave electromagnetic fields generated at the wave propagating surface interact with one or more analytes at the interface between the waveguide and a test medium. Test mediums may include a gas, liquid or solid. In one configuration, for example, the wave propagating surface is coated with a reactant coating complementary or specific to at least one analyte and with a reference material that absorbs light in a set of wavelength bands and emits light at a different set of wavelength bands. The reference material includes fluorophores, chemiluminescent materials, time resolved fluorophores and luminescent compounds. In one example, the reactant coating of the wave propagating surface binds at least one analyte which in turn binds a homologous binding partner carrying a fluorescent molecule. The binding partner and the reference material may be attached to the surface simultaneously or

sequentially by covalent or noncovalent means. In another configuration only an analyte binding partner is attached to the surface; with the reference material being added to the test medium. A fluorophore reference material and the fluorescent labelled molecule can be selected so that both are excited by a set of wavelength bands and yet each emit an output signal at a different wavelength. Appropriate filters, if necessary, for signal input and signal output would be utilized in the apparatus to practice the invention as described below. The two different emitted fluorescent radiation wavelength(s) reenter the waveguide and are detected to enable accurate determination of the presence or quantity of one or more analytes. These configurations and a number of alternative configurations are described in more detail below or will become apparent to those skilled in the arts.

As stated above, there are a number of different configurations of an evanescent wave sensor to provide one or more wave propagating surfaces. One configuration involves a planar plate such as a microscope slide. Alternatively, a second configuration involves a hollow waveguide; while the third may be one as described in the applicant's co-pending European Patent application of even date claiming priority from U.S. Patent application no. 07/712003 entitled "Evanescent Wave Sensor Shell and Apparatus" and; a fourth and preferred construction is that of a solid cylinder such as a waveguide, and more particularly an optical fiber.

There are several factors to be considered in selecting a composition material for the sensor. One factor is that the material must have an index of refraction greater than that of the intended medium to be analyzed. Additionally, it is desirable for the material to be optically pure and provide low attenuation of the radiation of interest. Silica glass is suitable for ultraviolet or visible radiation, plastics such as polymethylmethacrylate (PMMA), polystyrene, and polycarbonate are suitable for visible radiation, and fluoride glass or chalcogenide are suitable for near infrared radiation. Other organic polymeric materials such as silicones, acrylates, fluoroacrylates, and the like can also be used as the sensor composition material. It is also desirable for the material to have negligible inherent fluorescence at the radiation wavelengths of interest for assays involving fluorescence. Other polymers and glass compositions would become apparent to those skilled in the art for application to various embodiments of this invention.

Additionally, it is desirable for the composition material to have suitable surface properties for attachment of reactant coating(s), including binding partners and reference materials, to it or to be made suitable or amendable to modification to assist attachment. A reactant coating as used herein shall be understood to include the attachment by coating means of a molecule which is receptive to a complementary molecule in a test medium to form a binding pair. Binding pairs include antibody-antigen, biotin-avidin and such other known substances which have a homologous substance as are known in the art. Coating as used herein shall be understood to include specific and nonspecific reactions including noncovalent binding and covalent binding.

Analyte, as used herein, shall be understood to include any of a variety of chemical and biochemical substances. The use of the term analyte herein shall be understood to include both its singular tense and plural tense as appropriate. The analyte sources may include physiological, scientific and industrial (toxic and nontoxic) test mediums; where the presence, absence or quantity of the analyte in the medium is sought; and where, for example, analysis of a physiological analyte is relevant to diagnosis and treatment of a disease. PMMA is the preferred embodiment material not only for its optical purity and its injection molding characteristics, but also because it is hydrophobic which enables antibodies and other molecules to be attached or absorbed to the surface simply by bringing them in contact with the PMMA. Antibody as used herein shall be understood to include Fab antigen binding fragments, univalent fragments Fab' and bivalent fragments F(ab')<sub>2</sub>. For glass, it is desirable to provide either a hydrophobic surface or one amendable to covalent and noncovalent coupling chemistries.

An example of the present invention, in which a first fluorophore is attached to a surface 10 and a second fluorophore becomes attached to the surface, is shown in Fig. 1. In Fig. 1, a first bound antibody 12 binds analyte antigen 14 which in turn binds labelled antibody 16. The labelled antibody 16 is labelled by means of a fluorophore 18. In one example, the fluorophore 18 is B-phycoerythrin (BPE). A fluorophore reference material 20A, such as fluorescein, is attached to the surface 10.

Alternatively, the fluorophore reference material may be incorporated between two surfaces as shown in Fig. 7 by reference 20B; or within the evanescent zone as shown by reference 20C; or as a component or label of the reactant coating as shown by reference 20D.

Referring to Fig. 1 an excitation radiation input 22 is selected to excite emission within the evanescent wave zone from both the fluorophore reference material 20A-D and the fluorophore 18 on labelled antibody 16. In the example of Fig. 1, in which the fluorophores are BPE and fluorescein, a radiation input 22 of light having an excitation wavelength of 480 nm excites, via an evanescent wave 23, a first emission signal 24 from the BPE antibody label at a wavelength of approximately 576 nm and a reference material emission

signal 26 from the fluorescein reference material at a wavelength of approximately 520 nm to determine analyte and reference material, respectively. This example would utilize appropriate filters for signal input and signal output. By comparing the relative signal of the analyte emission signal 24 to that of the reference material emission signal 26, substrate variations and signal variations are normalized and/or calibrated.

5 A second embodiment of the present invention, in which a fluorophore reference material is attached to the waveguide surface 10 and two separate fluorophores are in solution and one attached to the surface is depicted in Fig. 2. In Fig. 2, a first attached antibody 12, binds with a first antigen 14 which, in turn, binds labelled antibody 16. As in the previous example, the labelled antibody 16 is labelled with a fluorophore 18 such as BPE. A fluorophore reference material 20A such as fluorescein is attached to the surface 10. Unlike  
10 the previous example, however, the embodiment shown in Fig. 2 includes a second attached antibody 30 as well as a corresponding second antigen 32 and second labelled antibody 34. The second labelled antibody 34 is labelled with a second fluorophore 36 such as, for example, tris (2,2'-bipyridyl) ruthenium II dichloride (referred to as ruthenium fluorophore herein).

As before, an excitation radiation input 22, see Fig. 2, is selected to be of a set wavelength band(s) that  
15 will provide an evanescent wave 23 to excite detectable emissions from the fluorophore reference material 20A and the fluorophores 18 and 36 on the labelled antibodies 16 and 34. In this example where the fluorophores are fluorescein, BPE and ruthenium, a radiation input 22 of light having an excitation wavelength of 480 nm stimulates the first emission signal 24 from the BPE antibody label at a wavelength of approximately 576 nm, a second emission signal 38 from the ruthenium antibody label at a wavelength of  
20 approximately 610 nm and a reference material emission signal 26 from the fluorescein reference material at a wavelength of approximately 520 nm. This example would utilize appropriate filters for signal input and signal output. As described above, the fluorophore reference material need not be attached to the surface.

A variation of the above example, in which the waveguide comprises a solid cylinder such as an optical fiber is depicted in Fig. 3. Cylindrical waveguide 50, Fig. 3, allows an assay of two or more analytes in a  
25 test medium and includes a reference material for normalization and/or calibration. Additionally, the waveguide surface is coated with avidin 58 to provide an initial coating surface. A first reactant coating includes a first antibody 54 which is biotinylated, (a biotin molecule 56 is attached to the antibody 54 to enable binding with the avidin 58). A second coating includes second antibody 64 which is biotinylated to bind with the avidin 58. The surface also contains a biotinylated reference material dye 60A bound to the  
30 avidin coating, such as fluorescein.

Alternatively, the reference material may be a component of the said cylinder as shown in Fig. 3 by reference 60B; or attached directly or indirectly to the surface as shown by reference 60C; or bound to the first antibody 54 as shown by reference 60D; or as bound to the second antibody 64 as shown by reference 60E; or depending on the type and format of the assay bound to the complementary or specific binding  
35 partner of the first or second antibody (not shown); or within the evanescent zone (not shown).

Referring to Fig. 3, the first antibody 54 binds with first analyte 68 which in turn binds a first labelled antibody 69 which is labelled with a fluorescent molecule 70. The second antibody 64 binds a second analyte 72 which in turn binds a second labelled antibody 73 carrying a fluorescent molecule 74. In one example, the first antibody 54 is anti-CKBB and the second antibody 64 is anti-CKMB. The first molecule 70  
40 is TEXAS RED<sup>R</sup> and the second molecule 74 is BPE.

The waveguide 50 is formed by coating the outer surface 51 with the avidin 58, or with strept-avidin, which is in solution at a concentration of approximately 200 micrograms per milliliter. The waveguide 50 is coated in a dilute solution of biotinylated fluorescein 60A. The waveguide then is coated in a solution containing biotinylated anti-CKBB at a concentration of approximately 50-200 micrograms per milliliter. In a  
45 preferred embodiment a calculated amount of the fluorophore reference material and biotin anti-CKBB are coated to the waveguide.

When fluorescent molecule 70 is TEXAS RED and the fluorophore reference material is fluorescein, two peak emission wavelengths can be detected at 615 nm and 520 nm, respectively. In the analysis of two or more labelled antibodies, according to the present embodiment, a third output wavelength at 576 nm, for  
50 example, can be detected when a second fluorescent molecule 74 is BPE. Appropriate filters would be utilized for signal input and signal output.

A configuration in which the fluorophore reference material is encapsulated, is depicted in Fig. 4. This configuration is particularly desirable in circumstances in which it is necessary to physically isolate the fluorophore reference material from the test medium. Thus, as shown in Fig. 4, a cylindrical sensor  
55 waveguide 120 has a surface coating 122 containing a fluorophore reference material 124. An antibody 126 for the antigen 128 to be detected is attached to the surface. As before, if it is necessary to enhance binding of the antibody 126 to the surface, binding partners, i.e. avidin-biotin and others known in the arts, can be employed. Labelled antibodies 130 for antigen 128 are contained in test medium L along with the

subject antigen 128. The labelled antibodies include fluorophores of the general type described previously, as well as those which would be utilized by those skilled in the arts. The antigen 128 is allowed to bind with antibody 126 and also with labelled antibodies 130. Detection of the antigen is carried out in the same manner as previously described.

5 Unlike the previous examples, however, in Fig. 4, the fluorophore reference material 124 is encapsulated within the surface coating 122 on the waveguide. Thus, undesirable interactions i.e. charge-charge interactions, between the fluorophore reference material 124 and the antigen 128 and/or the labelled antibody 130 are reduced. Ideally, the refractive index of the encapsulating medium should be less than the waveguide refractive index so that the reference light is also generated according to evanescent wave  
10 principles. As before, this embodiment is not limited to a system for detecting only a single analyte. Rather, multiple binding partners allowing detection of multiple analytes can also be employed.

Use of different fluorophores having selected absorption and emission spectra are illustrated in Figs. 5A-5C. These examples would utilize appropriate filters for signal input and signal output.

Fig. 5A illustrates two fluorophores which have overlapping absorption spectra  $A_1$  and  $A_2$ , such as for  
15 the dyes fluorescein and RPE. Both fluorophores can therefore be stimulated by a single excitation set of wavelength bands, indicate by arrow 370 between 480-490 nm in this example. The fluorescein has an emission spectra  $E_1$  which is different from emission spectra  $E_2$  of RPE, and therefore two different output signals are generated.

The use of two fluorophores having different absorption and emission spectra is illustrated in Fig. 5B.  
20 Neither the absorption spectra  $A_1$  nor the emission spectra  $E_1$  overlap with the second absorption spectra  $A_2$  or the emission spectra  $E_2$  of a second fluorophore. In one example, the first fluorophore is fluorescein and the second fluorophore is TEXAS RED.

The use of two fluorophores in which the emission spectra  $E_1$  overlaps the absorption spectra  $A_2$  of the second fluorophore is illustrated in Fig. 5C. One such set of dyes is fluorescein and BPE. Other  
25 combinations include BPE and TEXAS RED or C-phycocyanin and TEXAS RED.

Therefore, it can be seen that a number of dye combinations can be used with the embodiments of the present invention in the use of one or more wave propagating surfaces. In addition, a dye may be used as a reference material for itself and more than one dye may be used as a reference material. A partial listing of  
30 suitable dyes is illustrated in TABLE I. The use of alternate dyes and dye combinations to practice the invention as described herein will be apparent to those skilled in the arts.

Table I

35	ABBR	DYE <sup>1</sup>	APPROXIMATE (≥ 50% Abs RANGE) nm	EXCITATION WAVELENGTH nm	EMISSION WAVELENGTH nm
	FITC	Fluorescein isothiocyanate	475-505	493	520
40		Fluorescein	475-505	496	520
	RPE	R-Phycoerythrin	480-570	495, 536 566	576
	BPE	B-Phycoerythrin rhodamine B	500-570 500-585	546 578	576 604
45	TR	TEXAS RED <sup>R</sup>	580-610	596	615
	CPC	C-phycocyanin	570-640	620	650
	APC	Allophycocyanin	600-660	650	660
	RPC	R-phycocyanin	545-635	555, 618	642
50	RUTHENIUM	Tris (2,2'-bipyridyl) ruthenium II dichloride	410-490	450	610

<sup>1</sup> Source of dyes: Molecular Probes, Inc., Eugene, OR (U.S.A.)

55 This invention describes a sensor utilizing a reference material which provides sensor normalization and/or calibration, to compensate for variations between different sensors or variations that result from fluctuations in the radiation input and signal collection. Variations between different sensors are generally of three types: a) geometric irregularities in which the optical surfaces are not maintained in a perfectly parallel relationship, b) surface irregularities resulting from damage to the waveguide surface, and c) light input

variations due to the optical processor. Other variations between sensors include the age of the sensor and the use of the sensor. The reference material when used for normalization and/or calibration provide the following advantages: providing accurate and precise test results, the ability to test the effects of aging of the reactant coating, correlating substrate and coating variability between sensors in a given manufacturing batch/lot or assay, the ability to examine variations due to sensor manufacture or handling during coating, and of detecting defective sensors. Further applications of the reference material will become apparent to those skilled in the arts.

The effect of signal variations caused by surface irregularities is shown schematically in Figs. 6A and 6B. Referring to Fig. 6A, a sensor 150 having an increasing diameter is shown; while Fig. 6B shows a sensor 150 having a decreasing diameter. It is noted that the geometric variations depicted in Figs. 6A and 6B are grossly exaggerated for purposes of illustration only. Additionally, as used herein, the terms increasing and decreasing diameter for the sensor are intended to refer to the diameter of the waveguide progressing in the direction of the radiation input wave. The sensor has a fluorophore reference material 152 and an antibody 154 attached thereto. Test medium L contains an antigen 156 to be analyzed and a labelled antibody 158 which can bind antigen 156. A light input 160 produces an evanescent wave 162 at each region of contact 164 with the wave propagating surface. The evanescent wave 162 stimulates an emission signal 166 from the labelled antibodies 158 which become bound to antigens on the waveguide. The evanescent wave 162 also stimulates emission of a reference signal 168 from fluorophore reference material 152 attached to the waveguide. Alternatively, the fluorophore reference material may interact with the waveguide as described above.

The signal output of the evanescent waves 162 varies partially in proportion to the number of bounces (reflections) 164 with the waveguide surface. However, for a waveguide of increasing diameter, it is seen that the number of bounces will decrease per unit length as the light input 160 travels along the waveguide. This decreasing number of contacts results in a lower evanescent wave signal output further along the waveguide, thereby decreasing the emission signal resulting from the presence of the analyte toward the distal end of the waveguide. However, since both the signal indicative of the presence of the analyte 166 and the reference material signal 168 will be affected equally by the decreasing evanescent wave signal output, the ratio of the analyte emission signal output to the reference material emission signal output should remain constant for any given test medium. Thus, the use of the fluorophore reference material provides a means for compensating for variations among sensors in which the waveguide diameter increases.

Similarly, the fluorophore reference material provides a means to compensate for a waveguide having a decreasing diameter as shown in Fig. 6B. In Fig. 6B, the reference numerals represent the same components as defined in Fig. 6A. However, as shown in Fig. 6B, rather than decreasing the incidence of evanescent waves by a spreading of the contact points 164, the decreasing diameter causes the light input to experience increasing reflection angles at the contact points 164, ultimately resulting in a signal loss once the critical angle for internal reflection of the signal 160 is passed. This signal loss is shown as light component 170 which exits the waveguide at region 172. As shown in Fig. 6B, once the light input exits the waveguide, no evanescent waves are produced distal to the exit point. Accordingly, no signal indicative of the presence of the analyte 166 will be produced beyond the point at which the light input escapes the waveguide. Thus, the signal output of the analyte will be that produced by labelled antibodies that are attached to the waveguide in the evanescent zone. As in the previous example, since both the analyte signal 166 and the reference material signal 168 will be affected equally by the loss of the light input beyond a certain point on the waveguide, the ratio of the emission signal output to the reference material signal output should remain constant for any given test sample. As before, the use of the fluorophore reference material provides a means for compensating for variations among sensors in which the waveguide diameter decreases.

The effect of surface irregularities is shown schematically in Fig. 7. It is noted that a sensor having a surface defect of any significance should be rejected for use in analyzing a medium for the presence of an analyte. The sensor should optimally be pretested with a reference material before contact with a test sample. In the preferred embodiment the reference material will be attached to the sensor for pretesting purposes.

In Fig. 7, a waveguide 180 contains a surface irregularity 182. Light input 202 is internally reflected along the waveguide to produce evanescent radiation but the reflection is effected by the defects; and such effects are undesirable since they reduce the production of evanescent waves in the region distal to the defect, thereby decreasing the signal of the analyte emission signal 166 and the reference material emission signal 168. However, since these two signals are affected equally, their ratio will remain constant for a tested medium. Thus, the presence of the fluorophore reference material acts to normalize and/or



calibrate variations in signal input and signal collection.

Similarly, variation(s) in light input will not affect the ratio of the analyte emission signal to the reference material emission signal since both will vary equally with variations in the input. Thus, in this situation, the fluorophore reference material acts to normalize or calibrate the light input thereby reducing variation(s) in light input.

An optical processor as used herein shall mean any optical system that delivers excitation light to a sensor and collects the emitted light for subsequent signal processing. In the preferred embodiment, the optical processor is used for evanescent wave fluorescent measurements that measure more than one fluoromer present in the evanescent zone.

Referring to Figs. 8-10, the optical processor 300 and the system of Fig. 11 includes the following components:

- Light source - Any source or light that has the spectral characteristics to cause the excitation of all of the fluoromers of interest present in the Evanescent wave region; which is identified by reference numeral 310.
- Sensor - Any configuration of a device used for the purpose of fluorometric Evanescent Wave signal generation and measurement; which is identified by reference numeral 312.
- Coupler - An-appropriate optical device for the connection of the optical processor to the sensor; which is identified by reference numeral 314. In the approach here this coupler also is the device used for the collection of the signal being generated by the sensor
- Detector A - Any signal detection device optimized for Signal to Noise Ratio (SNR) for the spectrum that is transmitted by filter 3. Detector A is identified by reference numeral 316.
- Detector B - Any signal detection device optimized for SNR for the spectrum that is transmitted by filter 2. Detector B is identified by reference numeral 318.
- Element A - Dual band dichroic which reflects the excitation light of the fluoromers to be measured and transmits the emission signals of the two fluoromers being measured and which is identified by reference numeral 320. (It should be noted that any standard beam splitter can be used but would reduce the SNR).
- Element B - Dichroic that transmits the emission light of one of the fluoromers being measured and reflects the light emitted by the second fluoromer and which is identified by reference numeral 322. (It should be noted that any standard beam splitter can be used but would reduce the SNR).
- Filter 1 - Dual band interference filter for transmitting the excitation light for the two fluoromers to be measured; and which is identified by reference numeral 324.
- Filter 2 - Interference filter that transmits the light generated by one of the fluoromers; and which is identified by reference numeral 326.
- Filter 3 - Interference filter that transmits the light generated by the second fluoromer; and which is identified by reference numeral 328.

The following element are only used in Figs. 9, 10, and 11)

- Filter 4 - Interference filter that transmits the excitation light for the second fluoromer being measured; and which is identified by reference numeral 330.
- Filter 5 - Interference filter that transmits the excitation light for one of the fluoromers being measured; and which is identified by reference numeral 332.

The system as shown in figures 9 and 10 function as follows:

- A) Filter 1 filters the light source in such a way that allows for the excitation frequencies for FITC (Fluoresceinisoithiocyanite) and APC (Allophycocyanin) to be transmitted simultaneously in a way that these spectrum can be rejected by filters 2 and 3. This rejection is necessary to eliminate the detector's ability to detect the excitation light. This rejection is imperative to optimize the SNR of the system.
- B) Filters 2 and 3 are optimized to transmit the emitted light of APC and FITC respectively. See Omega Optical; Technical Report "Optical Coatings for Fluorescence Instruments; Aug. 1987; Omega Optical, Vermont; and Omega Optical; Technical Report "There's No End To Light - Optical Interference Filters and Coatings", 1987; Omega Optical, Vermont; both of which are incorporated herein by reference.
- C) Element "A" maximizes the reflection of the excitation light of APC and FITC which optimizes the system signal generation. This element at the same time maximizes the transmission of the emitted light and optimizes the signal measurement capability.
- D) Element "B" can separate the emitted light of the APC and FITC fluorometric signals in an efficient manner by reflecting the FITC light emitted and transmitting the APC light emitted.

The invention here is unique in that it is a multifrequency multimeasurement (more than one fluoromer) system. This optical processor's capabilities differ significantly from systems known in the art.

In view of the above disclosure, alternative designs of the components of the system may be provided including: the use of a single detector to measure more than one fluoromer; the measurement of the reference signal on a surface difference from the one in which the excitation light entered the surface; the use of a system with moving parts, i.e. moveable or interchangeable filters.

5 Although specific features of the invention are shown in some drawings and not in others, it is for convenience only as each feature may be combined with any or all of the other features in accordance with the invention.

It should be understood, however, that the foregoing description of the invention is intended merely to be illustrative thereof and that other modifications, embodiments and equivalents may be apparent to those skilled in the arts without departing from the spirit.

## Claims

1. An evanescent wave system for receiving one or more electromagnetic radiation input signals and emitting at least two output signals, said system comprising:
  - a waveguide having at least one wave propagating surface;
  - a reference material which interacts with said waveguide;
  - wherein the surface is capable of receiving one or more radiation input signals and causing an output emission radiation signal indicative of at least one analyte and an output emission radiation signal indicative of a reference material; and
  - wherein the reference material provides for normalization and/or calibration of the system.
2. The system of Claim 1 wherein the surface includes a reactant coating/coated thereon.
- 25 3. The system of Claim 2 wherein the reactant coating has attached thereto certain molecules including:
  - a) one or more antigens or antibodies; and/or
  - b) one or more amino acid sequences; and/or
  - c) one or more nucleic acid sequences; and/or
  - d) one or more receptors; and/or
  - 30 e) one or more binding partners or ligands.
4. The system of Claim 1 wherein the waveguide comprises two separate parallel surfaces in a conical shape.
- 35 5. The sensor of Claim 1 wherein the waveguide comprises a hollow waveguide.
6. The system of Claim 1 wherein the waveguide comprises an optical fiber.
7. The system of Claim 1 wherein the waveguide comprises a planar structure having opposed parallel surfaces.
- 40 8. The system of Claim 2 wherein the reference material enables the testing of the waveguide and reactive coating for effects of storage or aging.
9. The system of Claim 1, wherein the reference material enables the correlation of binding partner and coating variability between waveguides in a given manufacturing batch/lot or assay.
- 45 10. The system of Claim 1, wherein the reference material enables the examination of variations due to waveguide manufacture or handling during coating.
- 50 11. The system of Claim 1, wherein the reference material enables the detection of defective waveguides.
12. The waveguide of Claim 3 wherein the waveguide is transmissive to light which can excite emission of a labelled molecule and is transmissive to radiation from the labelled molecule.
- 55 13. The system of Claim 1 further including means for isolating the reference material from the analyte or molecular species.

14. The system of Claim 1 wherein the means for isolating comprises a solid material.
15. The system of Claim 14 in which said means for isolating comprises a material having an index of refraction substantially equal to the index of refraction of the test medium.
- 5 16. The system of Claim 13 in which said means for isolating includes a chemical layer.
17. The system of Claim 1 wherein said system further comprises means for contacting the waveguide with a medium or test sample;  
10 means for propagating light through the waveguide to generate evanescent wave electromagnetic fields at the surface to irradiate the test medium or test sample; and  
means detecting light reentering the waveguide and emitted from the waveguide surface.
18. The system as recited in Claim 17, wherein said filter includes at least one filter;
- 15 19. The system as recited in Claim 18, wherein said filter is a dual band interference filter which transmits the excitation signal for a reference material and at least one analyte to be measured.
20. An evanescent wave sensor for receiving one or more light input signals and emitting at least two output signals, comprising:  
20 a sensor having at least one wave propagating surface for sensor propagating light input along the surface; said surface including a reactant coating and a reference material; wherein the surface is capable of receiving a light input signals and causing a reference output signal indicative of the reference material and an output signal indicative of the presence of at least one molecule in a medium  
25 or test sample; and wherein the reference material provides normalization and/or calibration of the system.
21. A method for analyzing a test medium or test sample, comprising:  
a sensor having at least one wave propagating surface, said sensor propagating a light input along  
30 said surface;  
the surface having a reactant coating including a reference material and a binding partner of at least one analyte, said surface being capable of receiving the light input and causing a reference output signal indicative of the reference material at least one output signal indicative of the presence of at least one molecule or species and wherein the reference material provides normalization and/or  
35 calibration of the system.
22. The method of Claim 21 wherein said method further comprises contacting the waveguide with the test medium;  
propagating light through the waveguide to generate evanescent wave electromagnetic fields at the  
40 surface to irradiate the test medium; and  
detecting light reentering the waveguide and emitted from the waveguide surface.
23. The method of Claim 21 in which detecting includes monitoring at least a first output signal indicative of the presence of at least a first analyte and a reference output signal indicative of the presence of the  
45 reference material.
24. A method of attaching one or more binding partners and at least one reference material to an evanescent wave sensor and method comprising:  
means for covalently attaching said binding partner(s) and said reference material(s) to the sensor;  
50 or  
means for noncovalently attaching said binding partner(s) and said reference material(s) to the sensor.
25. A method of attaching one or more molecules and at least one reference material to an evanescent  
55 wave sensor said method comprising:  
means for covalently attaching said molecules; and  
noncovalently attaching said reference material to the sensor; or  
means for noncovalently attaching said molecules; and

covalently attaching said reference material to the sensor.

26. The method of Claim 24 wherein said method further comprises contacting the waveguide with a test medium or test sample;

5 propagating light through the waveguide to generate evanescent wave electromagnetic fields at the surface to irradiate the test medium; and

27. A method of coating one or more molecules and at least one reference material to an evanescent wave sensor said method comprising:

10 means for covalently attaching at least one molecule and noncovalently attaching at least one molecule and covalently attaching at least one reference material and noncovalently attaching at least one reference material to the sensor.

28. A method of attaching one or more binding partners and at least one reference material to an evanescent wave sensor said method comprising:

15 covalently attaching at least one binding partner; and noncovalently attaching at least one binding partner; and noncovalently attaching said reference material to the sensor; or

covalently attaching at least one binding partner; and noncovalently attaching at least one binding partner; and covalently attaching said reference material to the sensor.

20 29. A method of attaching one or more binding partners and at least one reference material to an evanescent wave sensor, said method comprising:

a. covalent bonding and/or;

b. noncovalent bonding.

25 30. A method of attaching one or more binding partners to an evanescent wave sensor, said method comprising:

a. covalent bonding and/or;

b. noncovalent bonding.

30 31. An optical processor for measuring one or more fluoromers present in an evanescent zone, said processor comprising:

a. a light source;

b. a sensor;

35 c. a coupler; for connecting the optical processor to the sensor;

d. one or more interference filters that transmits light for at least one fluoromer;

e. at least one detector which is optimized for SNR for the spectrum that is transmitted by a filter;

f. dual band interference filter for transmitting excitation light generated by the fluoromer(s); and

40 g. a dual band dichroic which reflects excitation light of at least two fluoromers to be measured and transmits the emission signals of the fluoromers to be measured.

32. An optical processor comprising:

a. multiband interference filter for transmitting excitation light for at least two fluoromers; and

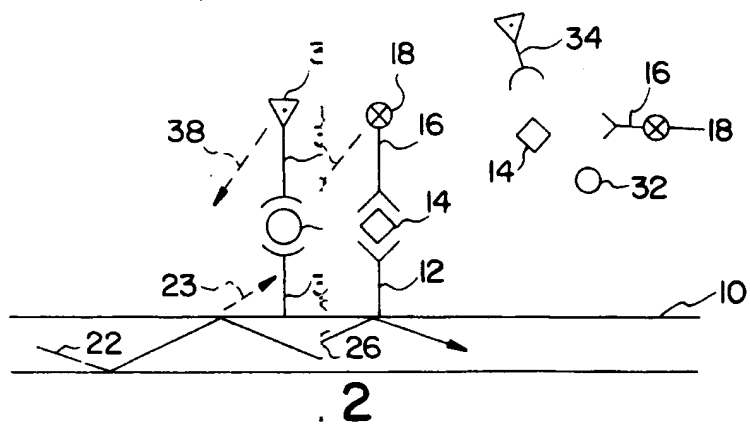
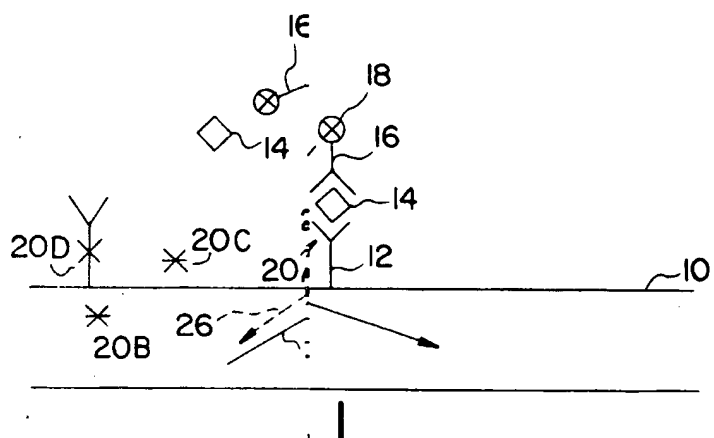
b. detector means for measuring the fluoromers

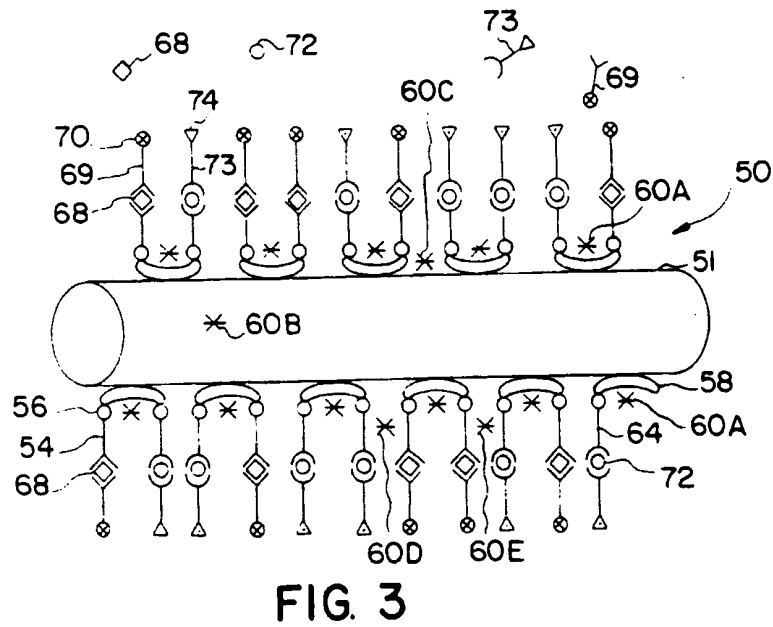
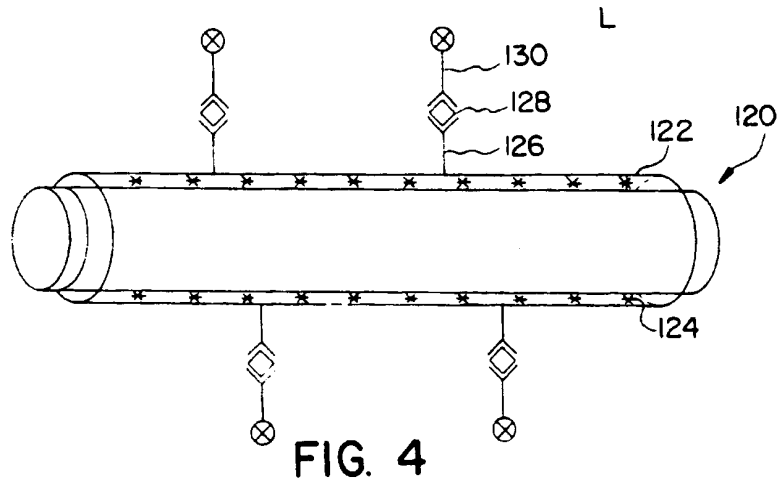
45 33. A multifrequency evanescent wave system comprising:

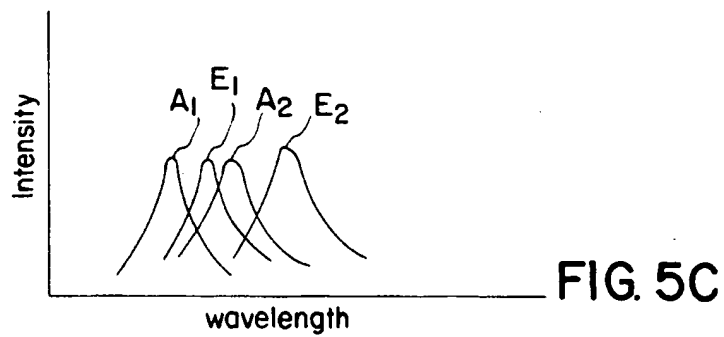
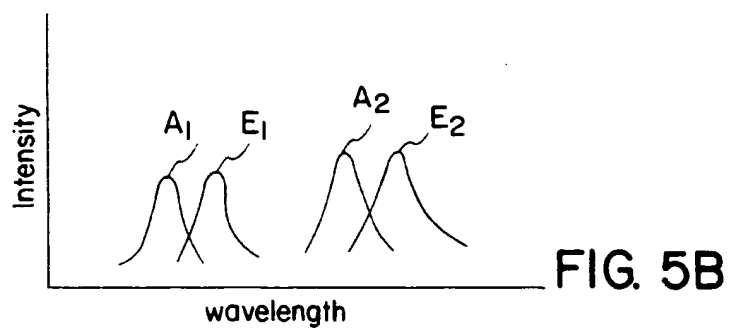
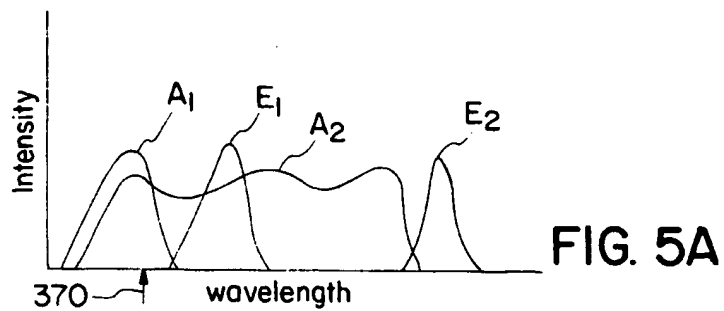
a. a signal source;

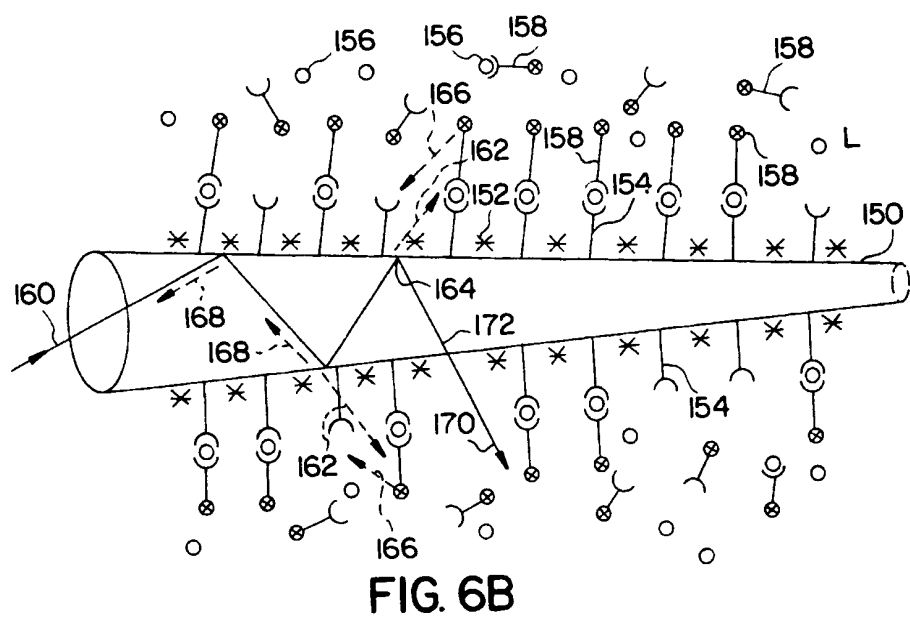
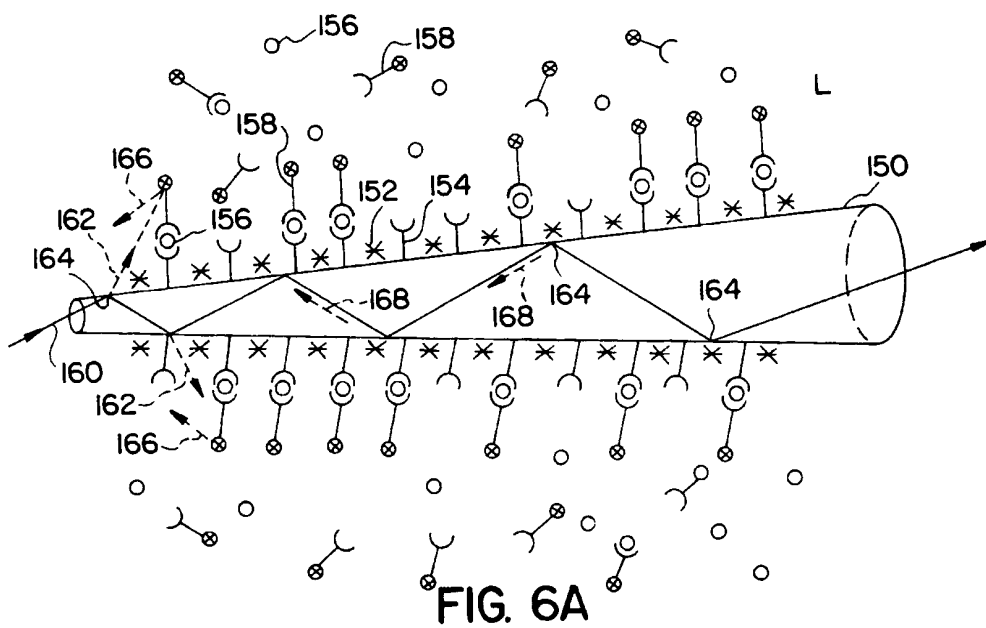
b. a sensor having interactive signal responsive components in an evanescent zone; and

50 c. an optical processor including a dual band dichroic which reflects excitation signal of the signal responsive components and transmits the emission signal to one or more detectors; and one or more signal detection devices.











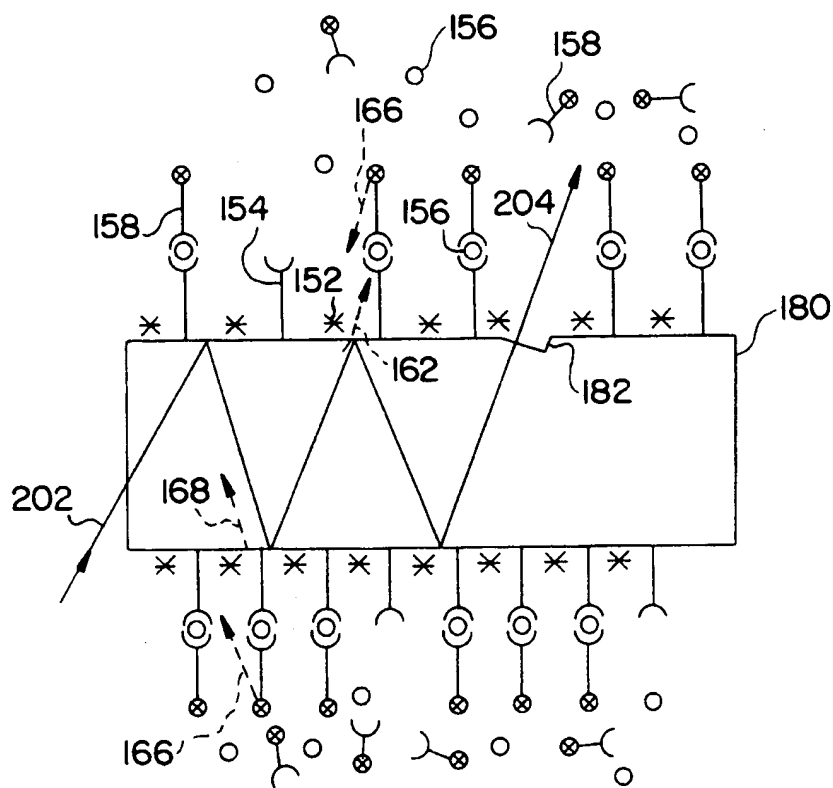


FIG. 7

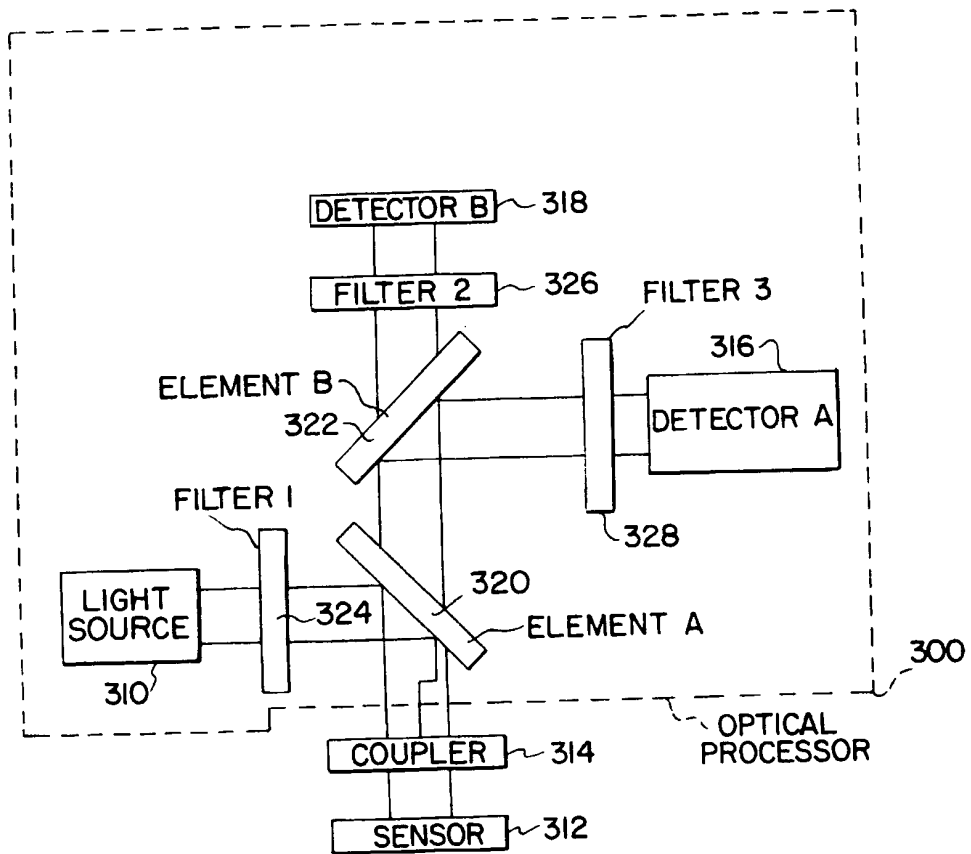


FIG. 8

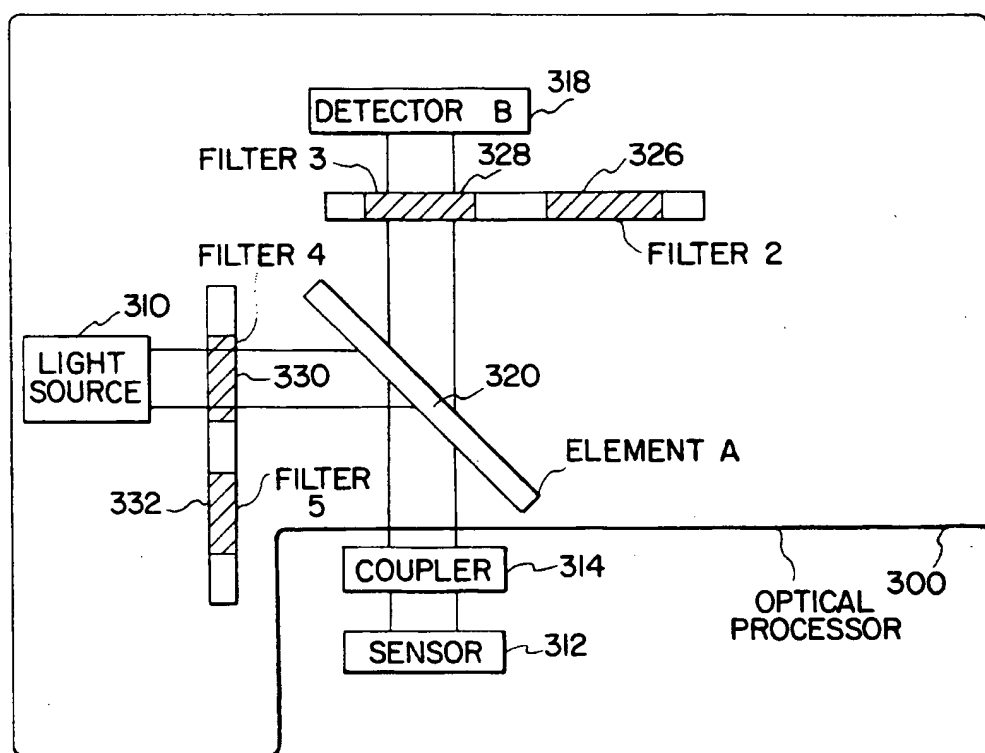


FIG. 9

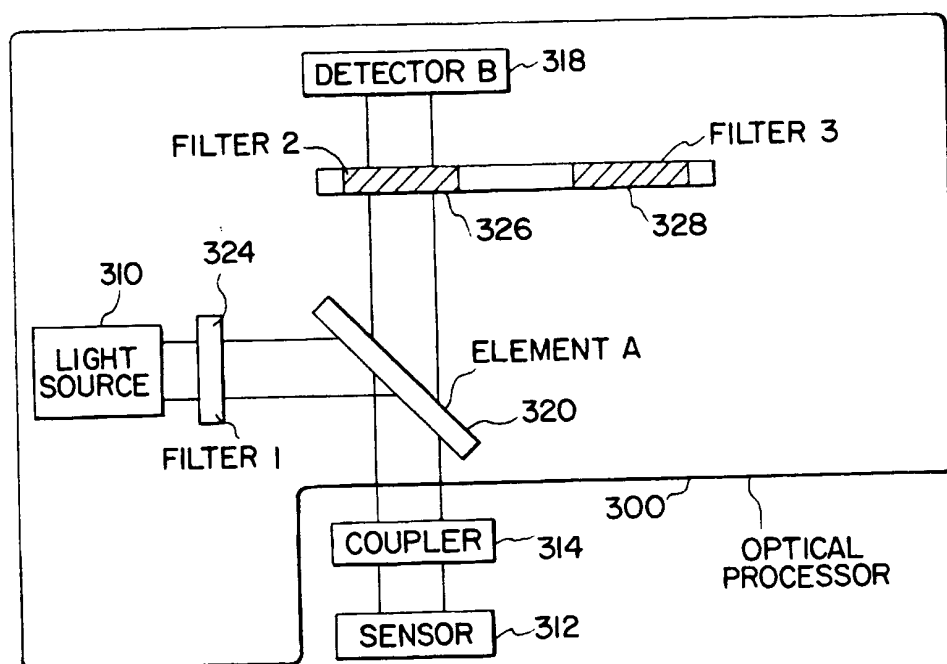


FIG. 10

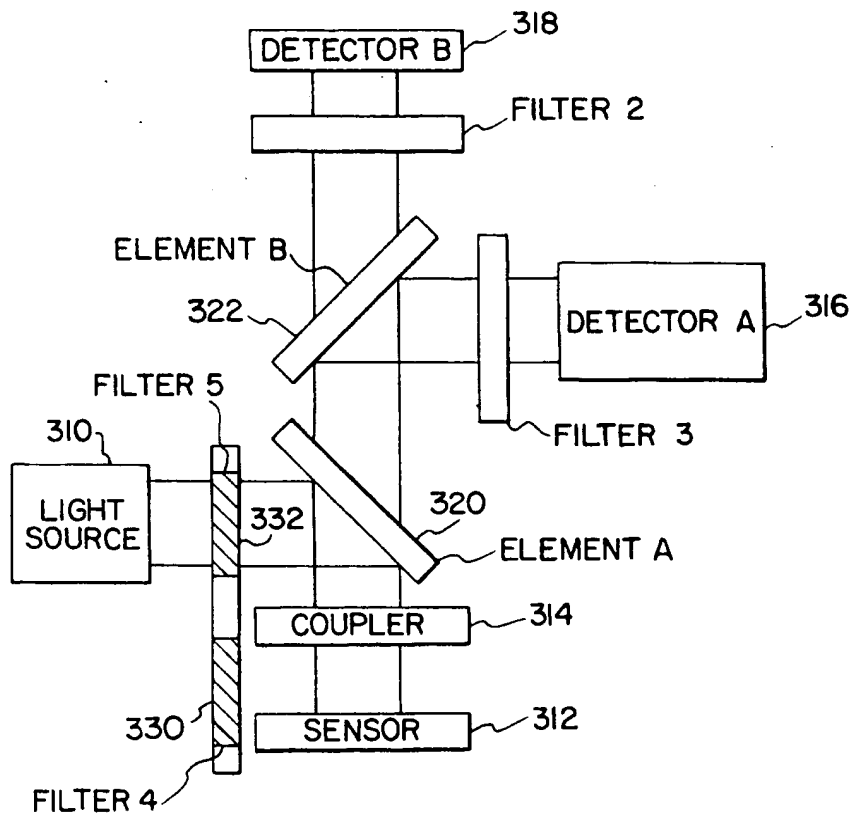


FIG. II



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 92305120.5
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	<u>US - A - 4 893 894</u> (F.M. CAIMI) * Totality * --	1,2,6, 17,22, 26	G 01 N 21/75 G 01 N 21/64 G 02 B 6/02
D,A	<u>US - A - 4 880 752</u> (D.B. KECK) * Totality * --	1,2,5, 6,17, 22,26, 33	
A	<u>US - A - 4 852 967</u> (T.A. COOK) * Abstract; column 4, lines 12-24; claims; fig. 4 * --	1,2,5, 6,7, 17,18, 22,26, 31	
A	<u>US - A - 4 818 710</u> (R.M. SUTHERLAND) * Abstract; column 10, line 42 - column 12, line 30; claims; fig. 2,4,5 * --	1,2,3, 6,12, 17,22, 26,31	
A	<u>US - A - 4 608 344</u> (T.J.N. CARTER) * Abstract; column 1, lines 7-26; column 8, line 1 - column 12, line 23; claims; fig. 4-18 * --	1,2,3, 6,12, 17,22, 26,31	TECHNICAL FIELDS SEARCHED (Int. Cl.5)  G 01 N 21/00 G 02 B 6/00
A	<u>EP - A - 0 211 587</u> (CORNING GLASS WORKS) * Totality * ----	1,2,3, 6,17, 22,26	
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 25-08-1992	Examiner ERBER
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document  T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons  & : member of the same patent family, corresponding document			